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MECHANISM OF ACTION OF NOVEL MARINE NEUROTOXINS ON ION CHANNELS

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INTRODUCTION

In the past few years we have seen a rapid advance in the elucidation of the mechanism of action of naturally occurring neurotoxins. The time span from the first isolation of a toxin to the understanding of its mechanism of action has been shortened more and more with each newly discovered toxin. This quickening pace has been driven by a widespread interest in the potent toxicants produced by marine organisms and aided by the application of highly sophisticated biochemical and pharmacological techniques. This review covers research on several marine neurotoxins with special reference to their recently discovered novel pharmacological actions. Some of the subjects in this area have already been reviewed. A review article by Narahashi (1) discussed the uses of chemicals as neurophysiological tools, including those of tetrodotoxin (TTX) and saxitoxin (STX). Similar subjects were later reviewed by Ritchie (2) and Catterall (3), and more recently by Pappone & Cahalan (4). Krebs (5) reviewed the recent developments in the field of marine natural products, giving comprehensive coverage of biologically active compounds. Baden (6) thoroughly reviewed the natural history of marine food-borne dinoflagellate toxins. Sea anemone toxins, brevetoxins, ciguatoxin, and palytoxin were discussed by Kaul & Daftari (7) in the context of bioactive substances from the sea. Ciguatoxin and maitotoxin were reviewed by Withers (8) from the medical perspective of ciguatera fish poisoning. Polypeptide neurotoxins from the sea anemone and the marine snail *Conus geographus,* as well as brevetoxins, were treated together with terrestrial neurotoxins in a chapter by Strichartz et al (9) on the modification of sodium channel gating.

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BREVETOXINS

A catastrophic episode of red tide in the Gulf of Mexico in 1946–1947 littered the beaches along the coast of Florida with tons of dead fish, with disastrous consequences to the region's economy and public health. More than 80 episodes of red tide have been recorded there since. The responsible organism was subsequently recognized as a new species of unarmored dinoflagellate and named *Gymnodinium breve* (10). This organism has subsequently been reclassified as *Ptychodiscus brevis* (11).

To date, a total of eight toxins has been isolated and purified from *Ptychodiscus brevis*. These toxins are now called brevetoxins, and a notation system, PbTx-1 through PbTx-8, based on the numbering system of Shimizu (12), has been proposed to designate the various brevetoxins isolated by several laboratories (13). The brevetoxins can be divided into two subclasses according to their chemical structures: PbTx-2, -3, -5, -6 and -8 belong to one group, and PbTx-1 and PbTx-7 to the other. We do not yet have information about the structure of PbTx-4. Current knowledge of the pharmacology of brevetoxin was obtained primarily from studies of PbTx-2 and PbTx-3.

Brevetoxins depolarize nerve and muscle membranes in a dose-dependent manner (14, 15). The extent of maximum depolarization is about 40 mV, and EC_{50} is 1.7 nM on crayfish giant axons. The depolarization of nerve terminals causes a massive release of transmitter, resulting in a wide range of responses in the effector organs. Thus the membrane depolarization and transmitter release can account for brevetoxin actions on a variety of organ systems as well as clinical symptoms of intoxication.

Early experiments suggested that the sodium channel was the site of brevetoxin action (16, 17, 18), and voltage clamp experiments provided direct demonstration of sodium channels as the target site of action (14, 17, 19). The channel modified by brevetoxin (PbTx-2 or PbTx-3) exhibits three striking characteristics (19): (*a*) The channel is activated at membrane potentials ranging from -160 to -80 mV, levels at which sodium channels do not normally open. (*b*) The channel is activated with an extremely slow kinetics; the time constant of activation is about 375–127 msec in the potential range from -80 to $+10$ mV. Thus the activation is about 1000 times slower than that of the normal sodium channel. (*c*) The channel is essentially devoid of fast inactivation. Thus the opening of sodium channels at large negative potentials and the absence of the fast inactivation process can account for the depolarizing action of brevetoxin.

Tritium-labeled PbTx-3 was prepared by Poli et al (13) by reducing PbTx-2 with tritium-labeled sodium borohydride NaB^3H_4. PbTx-3 binds with high affinity and specificity to rat brain synaptosomes. The specific binding is reversible and temperature-dependent, with K_D about 3 nM (at 4°C) and a

binding maximum of 6.8 pmol of toxin bound per mg of protein. According to studies of neurotoxin binding and neurotoxin-activated $^{22}Na^+$ flux, brevetoxin binds to sodium channels at a site different from the sites known for other neurotoxins (13, 20, 21, 22). Four distinct binding sites for neurotoxins in the sodium channel have been established (23). Neurotoxin-binding site 1, located on the external surface of the sodium channel (24), reversibly binds TTX or STX; these toxins inhibit Na^+ ion transport through the channel pore. Binding site 2, probably located within the channel, binds lipid-soluble toxins such as batrachotoxin and veratridine; these toxins cause persistent activation of the channel. Binding site 3, most likely an external site, binds polypeptide neurotoxins from sea anemone and α neurotoxin from the North African scorpion *Leiurus quinquestriatus*. These toxins inhibit the fast sodium channel inactivation mechanism. Binding site 4, presumably an external site also, binds β toxins from the American scorpions, such as *Centruroides sculpturatus*. The β scorpion toxins modify sodium channel activation rather than inactivation. Brevetoxin does not displace neurotoxins from these four binding sites. Conversely, brevetoxin binding is not displaced by these four classes of neurotoxins. Thus, brevetoxins appear to bind to sodium channels at sites other than sites 1–4.

Interestingly, PbTx-1 enhances the binding of batrachotoxin to site 2 (21). However, batrachotoxin has no effect on brevetoxin binding. This is due to the fact that batrachotoxin binds to activated or open channels with much higher affinity than to closed channels. Brevetoxin opens the channel, thereby enhancing batrachotoxin binding. On the contrary, brevetoxin binding is essentially independent of membrane potential, implying that it binds to open and closed channels equally well. Thus brevetoxin binding should not be enhanced by batrachotoxin; and such is the case.

CIGUATOXIN AND MAITOTOXIN

Ciguatera is a distinctive type of seafood poisoning resulting from the consumption of tropical fish that ordinarily are edible but at times become toxic. Although Halstead listed more than 400 species of fish implicated as ciguateric (25), the true figure may be considerably less than that. A survey of 527 cases of ciguatera in Queensland, Australia, between 1965 and 1984 shows that only slightly more than 26 species were incriminated; of these, the narrow-barred Spanish mackerel *(Scomberomorus commersoni)* accounted for most of the cases (26). In the United States, ciguatera fish poisoning has surpassed scombroid fish and paralytic shellfish poisoning as the most frequently reported food-borne disease. The problem is even more prevalent in the Caribbean and South Pacific. There are excellent reviews of research

findings on various aspects of ciguatera fish poisoning, particularly from the clinical perspective (8, 26, 27).

The major active principle of ciguatera poisoning, christened ciguatoxin, was first isolated by Scheuer and his colleagues in 1967 from the red snapper *(Lutjanus bohar),* the shark *(Carcharhinus menisorrha),* and the moray eel *(Gymnothorax javanicus)* (28). Recently, the origin of this toxin has been traced to a toxic dinoflagellate *Gambierdiscus toxicus* (29, 30, 31). A highly lipid-soluble compound, ciguatoxin has a molecular weight of 1,111.7 ± 0.3 and is believed to contain polyether moiety (32). The dinoflagellate also produces another toxin, the water-soluble maitotoxin, in more abundant quantity than ciguatoxin. Maitotoxin coexists with ciguatoxin in ciguateric fish. In fact, maitotoxin was first isolated from the surgeonfish, *Ctenochaetus striatus,* which is called "maito" in Tahiti. Much less is known about the chemical nature of maitotoxin than that of ciguatoxin. A third toxin, scaritoxin, isolated from the parrotfish, *Scarus gibbus,* is found to be interconvertible with ciguatoxin. An excellent summary of the pharmacology of these three toxins, both in vivo and in vitro, can be found in the review by Legrand & Bagnis (33).

Ciguatoxin at 0.2–1 nM induces a membrane depolarization and spontaneous action potentials in neuroblastoma cells and frog nodes of Ranvier (34, 35, 36). The effects are due to opening of sodium channels at the normal resting potential and to failure of the open channels to be inactivated during long lasting depolarization. The potassium channels are not affected. The sodium channels modified by ciguatoxin can be blocked by TTX. The reversal potential of the modified channel shows a large shift in the direction of hyperpolarization (by about 30 mV). Thus, ciguatoxin attacks the sodium channel specifically and modifies many properties of the channel. These effects strongly resemble those of brevetoxin.

Maitotoxin ranks among the most potent marine toxins. Its minimum lethal dose for mice is 0.17 μg/kg when injected intraperitoneally. It causes the calcium-dependent contraction of several smooth muscle and skeletal muscle preparations (37, 38). In cardiac muscles, maitotoxin has a positive inotropic effect at low doses (0.1–4 ng/ml); this effect is eliminated by Co^{2+} or verapamil (39, 40). Maitotoxin induces release of norepinephrine and dopamine from rat pheochromocytoma clonal cells (PC12) and a profound Ca^{2+} uptake by some cultured cell lines; both effects can be inhibited by inorganic and organic blockers of calcium channels (41, 42, 43). These results suggest that maitotoxin induces an increase in cell membrane permeability to calcium ions, influx of which triggers the release of transmitters and muscle contraction. Voltage clamp analysis by Yoshii et al (44) showed that, at the normal resting potential, maitotoxin induced a current that could be effectively blocked by verapamil or lanthanum. However, the induced current did not

flow through the voltage-activated calcium channels, because the currents through these channels decreased as the maitotoxin-induced current increased. The induced current showed an inward-rectifying property with a reversal potential of about −30 mV. The voltage clamp results suggest that maitotoxin creates a pore in the membrane with pharmacological properties similar to those of voltage-activated calcium channels. There are at least two possible mechanisms through which maitotoxin could accomplish this. First, the toxin may act as an ionophore or as an ion transporter. However, maitotoxin does not cause Ca^{2+} entry in rat liver mitochondria nor liposomes even at a high concentration of 10^{-7} g/ml (42). Thus this mechanism is deemed highly unlikely. The second possibility is that maitotoxin may transform a native protein in the membrane into a pore that allows Ca^{2+} to flow through itself. The effect may be analogous to that imputed to palytoxin, of transforming the sodium pump into a pore permeable to small ions (see below).

PARAGRACINE

Paragracine is isolated from the coelenterate species *Parazoanthus gracilis* (45). This compound and other related substances isolated from the Mediterranean species *P. axinellae* and *Epizoanthus arenaceus* are classified as zoanthoxanthins (46, 47). The basic structure of zoanthoxanthins contains a 7-carbon troponoid ring having two guanidine groups attached to it. Paragracine selectively blocks sodium channels without affecting potassium channels (48). The toxin blocks the channels only from the axoplasmic side and does so only when the sodium current flows in the outward direction. This block may be relieved by generating inward sodium currents, but as long as the channel is not opened by depolarization, the channel is kept blocked by the entrapped paragracine molecule. The blocking action does not depend on the absolute value of the membrane potential but depends on the value of the driving force for the sodium current, which determines the amplitude and direction of the sodium current. Thus the current-dependent block exhibited by paragracine is dependent on the outward direction of sodium current and is proportional to the amplitude of the outward current. Interestingly, there have been reports of similar current-dependent blocking of other channels, such as that by tetraethylammonium of potassium channels (49) and of guanidinium derivatives of acetylcholine-activated channels (50).

PALYTOXIN

Palytoxin is produced by coelenterate species belonging to the genus *Palythoa*. It was isolated independently by Attaway (51) from *P. cari-*

baeorum and *P. mammilosa* from Jamaica and the Bahamas, by Hashimoto et al (52) from *P. tuberculosa* from Okinawa, and by Moore and Scheuer from a species called *limu-make-o-Hana* ("deadly seaweed of Hana") in Hawaii, which was subsequently identified as *P. toxica* (53, 54).

The chemical structure of palytoxin has recently been elucidated (55, 56). Its molecular weight of 2680 far surpasses that of any other substances of natural origin without repeating units, and the complexity of its chemical structure ($C_{129}H_{223}N_3O_{54}$) is unprecedented. Even more remarkable is that despite its having 64 chiral centers, its absolute stereochemistry has also been established (54, 57). Because of its monstrous size and absence of repeating units, it is nothing short of miraculous that the structure of palytoxin has been determined at all, and that so much of its stereochemistry can be described. Palytoxin from different origins has been shown to be identical (54, 58).

Pharmacologically, palytoxin is the most toxic marine toxin known to date, having LD_{50} of 50–100 ng/kg for mice when administered by intraperitoneal injection (59). In rabbits, the most susceptible among the animals tested, the LD_{50} is even lower: 25 ng/kg. Palytoxin depolarizes every excitable tissue investigated including cardiac muscle (60, 61, 62, 63), skeletal muscle (61), smooth muscle (64, 65), and myelinated and unmyelinated nerve fibers (66, 67, 68). In the erythrocytes of susceptible species, palytoxin induces K^+ efflux followed by hemolysis (69). The depolarization and K^+ efflux are not due to the opening of existing ion channels, since TTX and nimodipine fail to antagonize palytoxin action (61, 67, 68, 70). Potassium channel blockers, such as apamin, quinine, and 4-aminopyridine, do not displace palytoxin from its binding (71).

In light of several kinds of evidence, Habermann and his colleagues have proposed that palytoxin interacts with Na, K-ATPase and converts the pump to an ion channel (70, 71, 72). The palytoxin-induced K^+ release is inhibited by ouabain in a noncompetitive manner. ATP potentiates palytoxin action. Dog erythrocytes, which lack Na, K-ATPase, are resistant to palytoxin action (70, 71, 72). In the crayfish axon, the palytoxin-induced depolarization, which requires intracellular ATP, is inhibited by ouabain (73). Garcia-Castineiras et al (74) showed that the crude extract of *Palythoa* inhibits the function of Na, K-ATPase, but they attributed this effect to a contamination of the extract by serotonin. Recently, using purified palytoxin, Ishida et al (75) and Böttinger & Habermann (76) were able to demonstrate that palytoxin indeed inhibits the action of the enzyme with IC_{50} of about 0.8–3.1 μM. Palytoxin does not bind to liposomes made from synthetic lipids and does not affect the membrane permeability of such liposomes (70, 77).

Palytoxin does not seem to bind to the same site as that associated with cardiac glycosides. Dog erythrocytes bind palytoxin; however, they are resistant to the toxin action (70). Kinetic data also indicate some qualitative and

quantitative differences between palytoxin and ouabain binding (70, 71, 75). Aglycones of the cardiac glycosides do not antagonize the palytoxin-induced K^+ release (78). It is likely that the binding sites for ouabain and palytoxin overlap to some extent.

The pores formed by palytoxin are selective for monovalent cations. The permeabilities of the ions relative to that of Na^+ are: Na:Li:Cs:NH_4 = 1:0.62:0.75:1.45 in squid axons (67), and Na:NH_4:guanidinium:tetramethylammonium = 1:1.72:1.11:0 in crayfish axons (73). In resealed erythrocyte ghosts, the pores are slightly permeable to inositol and sucrose but impermeable to inulin (72). Single channels formed by palytoxin in cardiac myocytes have a conductance of 10 pS. The channel undergoes state transitions between open and closed states, with the mean open time of 235 ms and mean closed time of 3.9 ms (79).

SEA ANEMONE TOXINS

The sting of sea anemones causes a wide range of symptoms including pain, skin welts, edema, itching, cardiac arrest, and paralysis. Their venoms contain mixtures of polypeptide toxins. The polypeptides can be divided into four major classes, according to their molecular weight. The two classes having the lower molecular weights (<3,000 and 4000–6000 daltons) have been found to modify the gating of sodium channels when applied externally in solution. The other two classes have no specific effect on ion channels.

Of all the sea anemone toxins, toxin-II from *Anemonia sulcata* (ATX-II) and anthopleurin-A from *Anthopleura xanthogrammica* (AP-A) are the most extensively studied (80). There is a striking sequence homology between these two toxins; they differ by only 7 amino acid residues out of a total of 47. They have essentially the same pharmacological actions on a variety of organ systems (81). Sea anemone toxins prolong the action potential in nerves and muscles, and they inhibit the gating of sodium channel inactivation (82, 83, 84, 85). They have no effect on potassium channels. Their potential usefulness in clinical treatment of congestive heart failure and myasthenic syndromes is being actively explored because of their positive inotropic action on cardiac tissues and stimulating effect on transmitter release at neuromuscular junctions. Both effects have been attributed to the action potential prolongation resulting from inhibition of the sodium channel inactivation mechanism (80, 81).

Interestingly, several toxins isolated from scorpions, e.g. *Buthus eupeus* and *Leiurus quinquestriatus,* also show pharmacological actions similar to those of sea anemone toxins, even though there is no sequence homology between these two groups of toxins (3). Not only do sea anemone and scorpion toxins have similar actions on sodium channel inactivation, but they

also enhance the persistent activation of sodium channels by lipid-soluble toxins such as batrachotoxin and veratridine. Their bindings are also similarly affected by membrane potential. Moreover, binding experiments in various preparations have demonstrated that sea anemone toxins competitively inhibit scorpion toxin binding. This observation led Catterall & Beress (86) to propose a common binding site associated with sodium channels on which these two classes of polypeptide toxins act. Presumably, some aspects of their tertiary structures permit them to interact with the same or overlapping receptor sites. Direct binding studies indicate that the binding reaction follows a simple Langmuir isotherm with a stoichiometry of one toxin to one receptor site (86, 87). Vertebrate neurons, cardiac muscle cells, and crustacean axons are sensitive to the action of sea anemone toxins. In contrast, giant axons of squid and cuttlefish are resistant to sea anemone toxins (82, 84). The binding site for the toxins is located on the external surface of the membrane. Estimate of the dissociation constant (K_D) for binding reaction ranges from 15 to 240 nM (88). The high affinity and specificity of these toxins makes them useful probes for investigations of the sodium channel.

Specific Action on Sodium Channels

The most prominent action of sea anemone toxins is inhibition of the gating of sodium channel inactivation. Voltage clamp analysis indicates that the toxins have no action on the gating of the sodium channel activation. This conclusion is also supported by fluctuation analysis. Before the advent of single-channel recording technique, Conti et al (89) took advantage of the non-inactivating current induced by the toxin to achieve a stationary state condition for measuring single channel conductance and activation time constant. The time constant determined by the noise measurements agrees well with the published activation time constant derived from the conventional relaxation analysis. Thus sea anemone toxins do not appear to affect the activation kinetics.

Because of its high potency and specificity for the sodium channel inactivation gate, the sea anemone toxin promises to become an important tool for probing the chemical property of the inactivation mechanism. As a polypeptide amenable to gentle protein modification, the sea anemone toxin is easier to modify than TTX or STX without loss of its activity. Because its action is reversible, the toxin is an alternative to proteolytic agents such as N-bromoacetamide or pronase as a means for studying the inactivation mechanism. Indeed, chemical modifications of ATX-II have been made to study the structure-activity relationship (90, 91). ATX-II has been radiolabeled, iodinated, and tritiated without loss of its biological activity (88, 92, 93). Labeling of ATX-II with the fluorescent probe (fluorescein-isothiocyanate), the phosphorescent probe (eosin-isothiocyanate), and the photoaffinity label

(4-azido-2-nitrophenyl) has been achieved (94, 95, 96). Thus sea anemone toxins and their various derivatives are being widely used for biochemical investigations of the sodium channel.

Interaction with Tetrodotoxin Binding

Romey et al (84) reported that pretreatment of crayfish axons with TTX prevented ATX-II from exerting its effect on the action potential. This result was attributed to the ability of TTX to prevent binding of ATX-II to the sodium channel. Similar findings were reported by Rathmayer (97) and Siemen & Vogel (98), who used other polypeptide toxins isolated from sea anemone nematocysts and scorpion venoms. In contrast, Low et al (85) demonstrated that TTX did not prevent AP-A from eliciting its effect on the action potential, observed after TTX had been washed out. This difference in the abilities of AP-A and ATX-II to produce an effect during TTX block is interesting in light of their extensive sequence homology and similar pharmacological actions. A recent study by Pernecky et al (99) indicates that there is no difference between the two toxins in this regard. ATX-II, like AP-A, gained access to the sodium channel in the presence of TTX. They also observed that the prolonged plateau phase of the action potential induced by both ATX-II and AP-A was more sensitive to the blocking action of TTX than the spike phase of the action potential. Thus after TTX had been washed from an axon treated with the sea anemone toxin in the presence of TTX, the spike recovered long before a plateau developed. As a result of this difference in the time course of recovery from blocking by TTX, apparently normal action potential emerged first when the axon resumed its excitability. A premature completion of the experiment immediately following action potential recovery from TTX block could lead to the mistaken conclusion that TTX prevents sea anemone toxins from exerting its effect on the action potential (84, 87). Additional washing of the axon with physiological saline solution is required to allow the slow recovery of the prolonged plateau from TTX block and to reveal the effect of sea anemone toxins on the action potential.

GONIOPORA TOXINS

Among the toxic species of corals, the stony corals *Goniopora* species have been shown to have considerable toxicity (100). A crystalline toxin was isolated and proved to be a peptide with a molecular weight of 12,000. The minimum lethal dose of the peptide was estimated to be 0.3 mg/kg by intraperitoneal injection. Microelectrode and voltage clamp studies on rabbit myocardium and crayfish giant axon showed that the toxin acted primarily on the sodium channel in a way resembling that of sea anemone toxins (101, 102, 103). In addition, *Goniopora* toxin increased nonspecific leakage con-

ductance that was insensitive to the blocking action of TTX. The exact nature of this leakage conductance is still unclear.

Recently another polypeptide toxin was isolated from a *Goniopora* species found in the Red Sea (104). This toxin has a M_r value estimated at 19,000 by SDS gel electrophoresis. It induces tonic contraction of guinea pig ileum, which is prevented by nitrendipine and (-)-desmethoxyverapamil, both being calcium channel blockers. The toxin inhibits the specific binding of (+)-^{3}H-PN 200-110 (a dihydropyridine antagonist) to the calcium channel protein of skeletal muscle T-tubule membranes. Furthermore, the toxin stimulates Ca^{2+} influx in chick cardiac cells in culture, which is inhibited by nitrendipine. These data suggest that this *Goniopora* toxin is a calcium channel activator.

CONUS TOXINS

Marine snails of the genus *Conus* contain one of the most virulent of animal venoms. The animals inject their toxin through a harpoon-like venom apparatus. All species of *Conus* are predaceous and can be divided into three feeding types: worm eaters (vermivorous), mollusc eaters (molluscivorous), and fish eaters (piscivorous). Only piscivorous species pose a serious threat to humans, and among this group, the venom of *Conus geographus* is considered the most dangerous. The earliest report of a human fatality from the sting of the species now known as *C. textile* was made by the great Dutch naturalist G. E. Rumphius, in his *D'Amboinsche Rariteitkammer* of 1705 (105). All venoms act as neurotoxins, but some vermivorous species possess venoms with tissue necrotic and hemorrhagic actions (106). A detailed study conducted by Kobayashi et al (107) on the actions of venoms of 29 species of *Conus,* encompassing all three feeding types, on the mouse diaphragm, guinea pig atrium and ileum, and rabbit aorta indicated that the venoms exhibited a great variety of actions. Indeed, the *Conus* venoms promise to be one of the great treasure houses of pharmacological agents. Through the use of protein chemistry techniques in conjunction with electrophysiological methodology, a number of toxins have been isolated that have a clearly identified mode of action, as shown in Table 1. A review by Cruz et al (108) offers detailed treatment of this subject.

At present there are two systems of nomenclature for naming the isolated toxins. The present authors fervently hope that a unified system will be quickly adopted before the situation degenerates into irremediable confusion.

The Japanese school generally adopts the species names of the binomial system for toxin names, as in the following examples: tessulatoxin from *C. tessulatus;* striatoxin from *C. striatus;* eburnetoxin from *C. eburneus;* and geographutoxin from *C. geographus*. Roman numerals are added to the name when isotoxins are found.

Table 1 Pharmacological actions of conotoxins

Species	Toxins	A.a.r[a]	M_r	Pharmacological actions	References
I. Piscivorous					
C. geographus	Conotoxin GI	13		Blocks ACh receptors	109, 110, 111
	Conotoxin GIA	15		Blocks ACh receptors	
	Conotoxin GII	13		Blocks ACh receptors	
	Conotoxin GIIIA (=Geographutoxin I)	22		Blocks muscle Na channels	113, 114, 115, 116
	Conotoxin GIIIB (=Geographutoxin II)	22		Blocks muscle Na channels	117, 118
	Conotoxin GIIIC	22		Blocks muscle Na channels	115
	Conotoxin GVIA	27		Blocks calcium channels	119, 121, 122
C. magus	Conotoxin MI	14		Blocks ACh receptors	112
	MTX		1,500	Opens Na channels	124
C. striatus	Striatoxin		25,000	Opens Na channels	125, 126, 127
II. Vermivorous					
C. eburneus	Eburnetoxin		28,000	Contracts rabbit aorta	128
C. tessulatus	Tessulatoxin		26,000	Contracts rabbit aorta	129
	Tessulatus-toxin		55,000	Increases Na^+ permeability Increases Ca^{2+} influx Contracts smooth muscles	130

[a]Amino acid residues

The Utah group uses the genus name as a prefix to form, for example, the generic name conotoxin which refers to all toxins isolated from *Conus* species. This is followed by a capital Roman letter to indicate species plus Roman numerals to distinguish various individual toxins. Examples are conotoxin MI from *C. magus,* and conotoxin GIV from *C. geographus.* Sometimes it is necessary to append a Roman letter to indicate isotoxins such as conotoxins GIIIA and GIIIB. When a physiological site of action is known, the Utah group also devises a system of classification to add to the name: α-conotoxins for acetylcholine (nicotinic) receptor toxins, μ-conotoxins for muscle sodium channel toxins, and ω-conotoxins for nerve terminal toxins.

α-*Conotoxins*

The conotoxins GI, GIA, and GII are the first toxins from more than 300 species of venomous Conidae whose amino acid sequences have been elucidated. These three homologous toxins, together with conotoxin MI, all consisting of 13–15 amino acid residues, act at the vertebrate neuromuscular junction. The α-conotoxin (GI, GIA, and GII) is specific for the acetylcholine receptor (109, 110). At a concentration of 25 μg/ml in Ringer's solution, the toxin blocks both the endplate potential and miniature endplate potential without affecting the action potential of either the nerve or the muscle (elicited by direct stimulation). The resting potential remained unchanged. The blocking effect on the endplate is fairly reversible. α-conotoxins competitively inhibit the binding of radiolabeled ^{125}I-α-bungarotoxin to the acetylcholine receptor (111). No electrophysiological experiments were performed on conotoxin MI from *C. magus;* its α-type pharmacological actions were inferred from its sequence homology to the other α-conotoxins (112).

μ-*Conotoxins*

Conotoxins GIIIA (geographaphutoxin I), GIIIB (geographutoxin II), and GIIIC, all comprising 22 residues, are isotoxins isolated from *Conus geographus* (113, 114, 115). This group of toxins preferentially blocks the sodium channels of skeletal muscle and *Electrophorus* electroplax, the latter tissue being also of muscular origin. The equilibrium dissociation constant of GIIIA at 0 mV and 22°C is 100 nM on single sodium channels isolated from rat skeletal muscle, reconstituted in lipid bilayer, and activated with batrachotoxin. Voltage clamp studies of skeletal muscles (116, 117) indicated that the kinetics of the macroscopic sodium current were probably not affected; the currents were proportionately depressed at all potentials in the presence of GIIIA. The voltage dependence of the mechanism of sodium channel inactivation was not affected by either GIIIA (116) or GIIIB (M. Kobayashi & C. H. Wu, unpublished observations). Thus, the actions of μ-conotoxin on

sodium channels are quite similar to the effects of TTX or STX. However, the physiological effects of μ-conotoxin on sodium channels are highly tissue-specific. No inhibition of batrachotoxin-activated sodium channels from brain membranes incorporated into lipid bilayers is observed at 1 μM GIIIA (115). GIIIB has no effect on the action potential of crayfish giant axons, guinea pig papillary muscles, and superior cervical ganglia up to 1–5 μM (117, 118). Binding data (118) confirm that μ-conotoxin binds to neurotoxin binding site 1 on the sodium channel. It blocks the binding of STX to this site with the K_D estimated to be 24 nM for T-tubules and 35 nM for homogenates of rat skeletal muscles. In contrast, the K_D for competitive inhibition of STX binding to rat brain synaptosomes is 2 μM. Thus, μ-conotoxin is the first neurotoxin to discriminate between the nerve and adult muscle sodium channels.

ω-Conotoxins

Conotoxin GVIA, consisting of 27 amino acid residues (119, 120), irreversibly blocks endplate potentials evoked by nerve stimulation (121). However, the spontaneous miniature endplate potentials remained unaffected. Neither the muscle nor the nerve terminal loses the ability to conduct action potential. Further analysis of the quantal content (121) indicated that conotoxin GVIA reduces neurotransmitter release by interfering with Ca^{2+} entry into the nerve terminal during the presynaptic action potential. The ω-conotoxin also eliminated the Ca^{2+} component of the action potential in dorsal-root-ganglion neurons from embryonic chicks. Ligand binding experiments (122) indicated that conotoxin GVIA binds specifically to high affinity sites on brain synaptosomes, and that the toxin-receptor complex is extremely stable. Competitive binding data demonstrated that this ω-conotoxin binds to calcium channels at a site distinct from those binding dihydropyridine, verapamil, and the diltiazem class of ligands. This toxin therefore provides a unique and potentially powerful probe for studying calcium channels and for exploring Ca^{2+} entry at nerve terminals.

It is noteworthy that the snail *C. geographus* ensures total muscle paralysis in its victims by cunningly engineering several neurotoxins to block the process of neuromuscular transmission at every step of the way. First there is ω-conotoxin that blocks transmitter release at the presynaptic site. Then there is α-conotoxin that blocks the acetylcholine receptor at the postsynaptic site. Lastly, there is μ-conotoxin that blocks the sodium channel of the muscle to prevent it from generating action potentials. In addition to those causing the neuromuscular block, there may be toxins in the venom that block the nerve action potential as well (123). Thus the snail produces toxins that act synergistically to immobilize its prey.

Other Conotoxins

Five additional toxins have been isolated from Conidae species. Their amino acid sequence has not yet been determined. Neither has their mechanism of action been firmly established. None of these toxins have any blocking action. Instead, they are characterized as having excitatory actions. The typical actions of each toxin are briefly summarized below.

A MYOTOXIN MTX Isolated from *C. magus* (124), this toxin elicited a complete loss of electrical response of the mouse diaphragm to electrical stimulation followed by a gradual rise in the muscle tone, an increase in the contractile force of the guinea pig atria, a tonic contraction of the guinea pig taenia caeci and powerful rhythmic contractions of the guinea pig ileum and vas deferens. These excitatory effects of the toxin were blocked by TTX, suggesting that these effects were due to an increase in Na^+ permeability of the cell membrane.

STRIATOXIN The venom of *C. striatus* yielded one major and five minor peptide peaks by gel filtration (125, 126). A cardiotonic glycoprotein was isolated from among the minor proteins. Named striatoxin, it has a molecular weight of about 25,000 as estimated by gel filtration. It causes a dose-dependent inotropic effect on guinea pigs' left atria, and the effect is completely inhibited in the presence of 1 μM TTX, suggesting that striatoxin opens the TTX-sensitive sodium channels of the myocardial cells. Striatoxin may be one of the major principles in the venom toxic to fish. Strichartz et al (127) found that an aqueous extract of the venom produced repetitive firing in response to a single stimulus applied to frogs' sciatic nerves. Subjected to chromatography on Sephadex G-50, the venom yielded seven peaks, two of which modified action potentials. One peak produced repetitive firing; the other a slow-decaying plateau of the action potential. Voltage clamp analysis of the crude venom (127) showed three distinct effects: a hyperpolarizing shift of the sodium channel activation; a slowing of the sodium channel inactivation; and a marked increase in the delayed potassium conductance.

EBURNETOXIN The venom of *C. eburneus* was found to cause a marked contraction of the rabbit aorta, which was antagonized by verapamil (128). The active principle, named eburnetoxin, has a molecular weight of 28,000 and causes a marked contraction of the aorta with a minimum effective dose of 8 nM. The contraction induced by eburnetoxin is inhibited by verapamil.

TESSULATOXIN Tessulatoxin was isolated from *C. tessulatus* by essentially the same procedure as that used for eburnetoxin (129). This toxin, with a molecular weight of 26,000, has the same biological activities as eburnetoxin.

The toxin-induced contraction of rabbit aorta was effectively inhibited by verapamil or a Ca-free solution.

TESSULATUS TOXIN A toxin causing smooth muscle contraction was isolated from *C. tessulatus* by Schweitz et al (130). More than twice as large as tessulatoxin, it has a molecular mass of 55,000 daltons and appears to be constituted of two distinct subunits of 26,000 and 29,000 daltons. The contraction induced by the toxin was prevented by pretreatment with the calcium channel blockers, nitrendipine or (−)-desmethoxyverapamil. The toxin caused a large increase in the initial rate of Ca^{2+} uptake by cardiac cells, but this uptake was insensitive to the calcium channel blockers. Voltage clamp experiments showed that the calcium currents are unaffected by the toxin. The toxin-induced Ca^{2+} uptake was inhibited by dichlorobenzamil and was suppressed when Na^+ was replaced by Li^+, suggesting the involvement of the Na^+/Ca^{2+} exchange mechanism. The toxin also increased the initial rate of Na^+ uptake by cardiac cells. It appears that the increase in Ca^{2+} uptake is secondary to the Na^+ loading. Thus the sequence of events appears to be initiated by the toxin-induced Na^+ influx, which leads to depolarization of cell membranes. The latter in turn triggers the opening of calcium channels and, with Ca^{2+} entry, the contraction of smooth muscle. In the cardiac cells, the Na^{2+} loading induced by the toxin activates the Na^+/Ca^{2+} exchange mechanism, resulting in Ca^{2+} uptake that is insensitive to the calcium channel blockers.

The exact mechanism by which the toxin induces Na^+ uptake has yet to be elucidated. Possible candidates for the Na^+ entry system, such as the voltage-dependent sodium channels, the epithelial amiloride-sensitive Na^+ channel, the Na^+/H^+ exchange system, and the $Na^+/K^+/Cl^-$ cotransporter, have been ruled out because specific blockers of each system have no effect on the toxin-induced Na^+ uptake.

Two minor peptides have been isolated from *C. geographus*. One is designated GIV and has an apparent molecular weight of 13,000 (131). It had no detectable toxicity to mice on intraperitoneal injection, but was a potent convulsant following intracerebral injection. The second peptide, designated GVA, is 17 amino acids in length (132). It elicited no obvious effects when injected intraperitoneally into either mice or fish. However, upon intracerebral injection in mice, it induced a sleep-like state. Since the snail does not normally deliver venom by intracerebral injection, the function of these two peptides remains unclear.

FUTURE PROSPECT

Much interesting new insight into the molecular details of acetylcholine receptors and sodium channels has been obtained through the application of

powerful neurotoxins. The marine neurotoxins described in this review show novel pharmacological actions with high binding affinity to ion channels and offer new experimental approaches for studying ion channels and receptors of excitable cells. Much has been achieved with only the handful of toxins we have now. How much more remains to be discovered is beyond our fathoming. Isaac Newton knew this same feeling and expressed it in the following words more than two and half centuries ago:

I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, while the great ocean of truth lay all undiscovered before me.

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